Role of free radicals and antioxidants in the pathogenesis of the inflammatory periodontal diseases

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**Introduction**

The human inflammatory periodontal diseases are amongst the most common of chronic diseases to affect adults. In the UK, 69% of adults have early signs of disease and only 5% are completely free from clinical signs of inflammation.\(^1\) The periodontal complex comprises alveolar bone, periodontal ligament, root cementum, and the overlying gingival (gum) tissues (fig 1). Gingivitis may be defined as “an inflammatory lesion, mediated by host-parasite interactions, that is confined to the gingival tissues”. The major cause of gingivitis is an accumulation of microbial plaque in and around the dento-gingival complex, which, when removed, results in complete resolution of the inflammatory lesion.\(^2\) Periodontitis is regarded as “an inflammatory lesion, mediated by complex host-parasite interactions, that leads to the loss of connective tissue attachment to root surface cementum and adjacent alveolar bone”. There are many forms of periodontitis and the changes associated with periodontitis are irreversible, resulting in tooth loss and substantial morbidity in medically compromised patients, where a focus of infection and subsequent bacteraemia may present a major risk.

The mouth possesses a unique hard/soft tissue barrier, that separates the internal systems from the external environment. The barrier (fig 1) is called the junctional epithelium and is permeable to external (bacterial) material passing into the adjacent connective tissues and blood stream, and to products of internal defence systems (leucocytes, complement, antibodies, pro-inflammatory cytokines, etc.) passing outwards. To assist this vulnerable barrier in protecting the underlying host tissues from damage by products of bacterial plaque, a fluid, gingival crevicular fluid (GCF), is produced from beneath the gingival margin. In health, GCF is a serum transudate containing all components of serum and also polymorphonuclear leucocyte cells, but during disease many products of the host-parasite conflict enter the fluid, which becomes a true exudate. GCF may be collected non-invasively on paper strips\(^4\) (fig 2), providing an ideal medium in which to study the complex bacterial–host interactions (fig 3) that characterise other similar inflammatory disorders.

Substantial data are available in the literature on the role of reactive oxygen species (ROS) and antioxidants in disorders such as the inflammatory lung diseases and in chronic immune mediated conditions such as rheumatoid arthritis. However, remarkably little information is available on the periodontal diseases, which show many of the pathological features of other chronic inflammatory diseases. The periodontal tissues also provide an ideal medium within which to study mechanisms of ROS mediated tissue damage and of antioxidant defence in response to bacterial colonisation, through the non-invasive collection of GCF. This paper, therefore, attempts to review current knowledge of free radical damage and antioxidant defence systems in inflammatory diseases, and to use the inflammatory periodontal diseases as a focus for discussion. Emphasis is placed upon the presence of low molecular weight thiols such as reduced glutathione (GSH) and cysteine in fluids that bathe vulnerable epithelial surfaces, and it is postulated that new therapeutic pathways may be found by using cysteine and GSH preserving drugs—for example, N-acetylcysteine, or indeed, inhibitors of the nuclear transcription factor NF-κB.

**Inflammatory cells and reactive oxygen species**

There is good evidence arising from studies of defective neutrophil function in Chediak-Higashi syndrome,\(^5\) ataxias,\(^6\) Job's syndrome,\(^7\) and chronic granulomatous disease,\(^8\) where profound tissue inflammation can lead to periodontal destruction, that the polymorphonuclear leucocyte has a protective role in the periodontal environment. However, evidence is emerging from several studies\(^9-10\) that in early onset forms of periodontitis, polymorphonuclear leucocytes are functionally activated and exhibit increased free radical production as measured by luminol dependent chemiluminescence. Indeed, peripheral blood monocytes from patients with periodontitis demonstrate higher prostaglandin E\(_2\) production upon stimulation than those from patients
radical species, which may be defined as any species capable of independent existence that contains one or more unpaired electrons. In recent years the term reactive oxygen species has been adopted to include molecules such as hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl) and singlet oxygen ($^1$O$_2$), which, while not radicals in nature, are capable of radical formation in the extra- and intracellular environments. Reactive oxygen species cause tissue damage by a variety of different mechanisms:

- lipid peroxidation (through activation of cyclooxygenases and lipooxygenases);
- DNA damage (base hydroxylations and strand breaks);
- protein damage, including gingival hyaluronic acid and proteoglycans;
- oxidation of important enzymes—for example, anti-proteases such as α-1-antitrypsin; and
- stimulation of pro-inflammatory cytokine release by monocytes and macrophages, by depleting intracellular thiol compounds and activating nuclear factor κB (NF-κB).

Most reactive oxygen species have extremely short half-lives (10$^{-9}$ to 10$^{-6}$ seconds), but they can cause substantial tissue damage by initiating free radical chain reactions. There are exogenous sources of ROS such as cigarette smoke and ionising radiation. Endogenous production is, however, more pertinent to the pathogenesis of periodontal disease and arises either accidentally due to leakage of electrons from their carriers within the respiratory chain of mitochondria passing directly onto oxygen, or functionally through the generation of oxygen radicals by phagocytes (fig 4). The latter process is thought to be implicated in the destruction of the connective tissues of the periodontium.

### THE SUPEROXIDE ANION

Superoxide (O$_2^-$) is formed chemically by the addition of an extra electron to oxygen (where * signifies an unpaired electron):

$$\text{O}_2 + e^- \rightarrow \text{O}_2^-$$

Polymorphonuclear leucocytes and macrophages (and to a lesser extent eosinophils, lymphocytes and fibroblasts) are examples of cells that produce superoxide as an antibacterial agent. Production in the polymorphonuclear leucocyte is the result of the so-called membrane bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase shunt (or hexose monophosphate shunt). This shunt fails to work in chronic granulomatous disease (CGD), which is why neutrophils from patients with CGD can engulf opsonised bacteria, but are unable to kill certain strains which are subsequently released in a viable state.

$$2\text{NADPH} + 2\text{O}_2^- \rightarrow 2\text{NADP} + 2\text{H}^+ + 2\text{O}_2^-$$

Superoxide is regarded as a weakly reactive radical, relative to the hydroxyl radical, but can nevertheless attack a number of biological tar-
The role of catalase in the extracellular environment is performed by a very important enzyme called glutathione peroxidase (GSH-Px), which is largely selenium dependent and reduces H₂O₂ whilst oxidising reduced glutathione (GSH) to its oxidised form (GSSG).

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

**THE HYDROXYL RADICAL.**

The hydroxyl radical is the most reactive radical known to man and interacts with most biological molecules. It can be formed from superoxide through the iron catalysed Haber-Weiss reaction, or from hydrogen peroxide through another transition metal dependent reaction called the Fenton reaction. This reaction is largely dependent upon Fe²⁺ and Cu²⁺ ions, and thus proteins that sequester iron or copper (for example, albumin, caeruloplasmin, haptoglobin, lactoferrin, and transferrin) are extremely important antioxidants.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH}^-
\]

The hydroxyl radical can stimulate a classic free radical chain reaction known as lipid peroxidation. When the hydroxyl radical is generated close to membrane phospholipids, it attacks the lipid side chains to form radical intermediates called peroxyl radicals (RO₂⁻), hydrogen peroxide and lipid hydroperoxides. Arachadonic acid is a preferential target for the hydroxyl radical. The accumulation of hydroperoxides can disrupt membrane function and the hydroperoxides can decompose to form cytotoxic aldehydes. End products of such lipid peroxidations include a unique class...
Mechanisms of preventing hydroxyl radical induced tissue damage include the binding of transition metal ions by the "preventative antioxidants" albumin, caeruloplasmin, haptoglobin, lactoferrin, and transferrin. Scavengers of the hydroxyl radical include vitamin C, uric acid and thiols, such as reduced glutathione and cysteine.

HYDROGEN PEROXIDE

Hydrogen peroxide can be produced by periodontal bacteria, by phagocytes from the NADPH oxidase shunt and also after dismutation of superoxide. While only a weak oxidant, it is has high potential to produce damage due to its ability to diffuse freely across cell membranes and undergo Fenton reactions with transition metals, thereby giving rise to site directed or site specific generation of 'OH. It has been proposed that hydrogen peroxide may act as a metabolic signal by oxidising protein thiol groups and triggering intracellular events. An example would be the oxidation of an important nuclear regulatory protein NFκB, a process thought to be responsible for the expression of HIV genes.

The activity of hydrogen peroxide on NF-κB is also responsible for the transcription of several pro-inflammatory cytokines of importance to periodontal disease pathogenesis, including interleukin-2 (IL-2), IL-6, IL-8, β-interferon, and tumour necrosis factor α (TNFα). NFκB activation (fig 5) can also be caused by bacterial endotoxins, IL-1 and TNFα. The process is rapid, as NFκB exists in the cytoplasm of most inflammatory cells, in a complex with an inhibited form (I-κB). Cytokines such as TNF-α and IL-1 are able to activate NFκB via protein kinase C and other kinases, which phosphorylate the I-κB part of the cytoplasmic complex, thereby releasing free NFκB rapidly and without the need for lengthy protein synthesis. The free NFκB diffuses from the cytoplasm, across the nuclear membrane and binds to DNA, stimulating the transcription of mRNA for the various proinflammatory cytokines. Recently, it has been shown that bacterial lipopolysaccharide (LPS/endotoxin) can also activate macrophage NFκB and subsequent cytokine transcription in a protein kinase C independent manner. As IL-1 and TNFα positively regulate their own production through the NFκB system, it is possible that the additive effects of endotoxin mediated cytokine production and that arising from the respiratory burst of polymorphonuclear leucocytes in response to the same organisms, could lead to substantial periodontal inflammation and subsequent tissue destruction.

Hydrogen peroxide is removed from cells by the action of antioxidant enzymes—for example, catalase, selenium dependent glutathione peroxidase and some other peroxidases.

HYPOCHLOROUS ACID

Hypochlorous acid is a powerful antibacterial agent and is also capable of causing oxidation of plasma membrane thiol (SH) groups and disruption of certain protein functions, even...
at concentrations as low as 10–20 µM. Such disruptions include the inactivation of glucose and amino acid transport systems and the K⁺ ion pump. Cell lysis occurs at higher hypochlorous acid concentrations and hypochlorous acid is capable of oxidising α-1-antitrypsin²⁷ and is also reported to activate neutrophil collagenase.⁵⁵ In the presence of amino acids hypochlorous acid reacts to form chloramines, which in turn protect cells from the former.⁵³ Hypochlorous acid is removed by reaction with the scavenging antioxidants albumin and ascorbic acid.³⁹

**SINGLET OXYGEN**

Singlet oxygen (⁰₂) is not a radical as it does not contain an unpaired electron. It is formed by an input of energy to O₂, which results in the reversal of spin direction of one of the outermost unpaired electrons, from a parallel spin to an opposing spin direction. This renders the singlet oxygen molecule unstable and more capable of oxidising other molecules. It is highly reactive with membrane lipids to produce peroxides, but information about its role in tissue damage is limited, and its role in periodontal inflammation is unknown.

**Antioxidant mechanisms**

The body contains a number of protective antioxidant mechanisms, whose specific role is to remove harmful oxidants as they form, or to repair damage caused by ROS in vivo. Antioxidants may be regarded as those substances which when present at low concentrations, compared with those of an oxidisable substrate, will significantly delay or inhibit oxidation of that substrate.⁴⁵

Antioxidants are classified according to their mode of action (table 1). Important antioxidants include: (1) the chain breaking or scavenging antioxidants: vitamin E (α-tocopherol), vitamin C (ascorbic acid), vitamin A (β-carotene), urate, bilirubin, and those substances containing thiol groups; (2) preventative antioxidants: these function largely by sequestering transition metal ions and preventing Fenton reactions and are therefore largely proteins by nature (for example, albumin, transferrin, lactoferrin, caeruloplasmin, haptoglobin, and ascorbic acid); and (3) enzyme antioxidants: these are enzyme systems that function by catalysing the oxidation of other molecules (for example, SOD, catalase and glutathione peroxidase).

The amount of information available relating to the importance of the body’s antioxidant systems in protecting against such damage is substantial²³⁴⁴ and yet only two studies⁵³⁵⁷ have investigated total antioxidant defence within biological fluids in relation to periodontal disease. Many antioxidants function by more than one mechanism. The enzyme based systems, including SOD, catalase and glutathione peroxidase have been mentioned previously and further review will be limited to those extracellular antioxidants of perceived importance in periodontal disease.

**CAROTENOID (VITAMIN A)**

Carotenoids, such as β-carotene, have long double bonds to attract and quench radical attack,⁵⁸ but currently little is known about the interactions of carotenoids and reactive oxygen species. Vitamin A deficiency has been implicated in periodontal destruction with animal models but results have not been reproduced in humans.

It is unlikely that individual vitamin deficiencies have significant detrimental effects on the periodontium, but the combined effects of vitamins A, C and E have potential as therapeutic adjuncts in view of their powerful antioxidant activities.

**ASCORBIC ACID (VITAMIN C)**

Ascorbate protects against oxidants present in cigarette smoke⁴¹ and is a powerful scavenger of hypochlorous acid, superoxide, singlet oxygen, and hydroxyl radicals. It also possesses the ability to regenerate α-tocopherol from the tocopherol radical that forms at membrane surfaces. GCF concentrations of ascorbic acid have been reported to be three times higher than those of plasma⁴⁵ and it has been shown to prevent activation of neutrophil derived collagenase⁵⁴ in GCF. In rheumatoid arthritis⁵⁶ plasma ascorbate concentrations are low but there is little evidence for any relation between plasma ascorbate concentrations and inflammatory periodontitis.⁴¹ though increased gingival bleeding is a common result of ascorbate depletion.⁴²⁴³

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**Table 1 Important extracellular antioxidants, their modes of action, solubility, and locations**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Mode of action</th>
<th>Solubility</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (vitamin C)</td>
<td>Chain breaking (scavenging)</td>
<td>Water soluble</td>
<td>Plasma, saliva, GCF</td>
</tr>
<tr>
<td></td>
<td>Preventative (binds metal ions)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regenerates α-tocopherol</td>
<td>Lipid soluble</td>
<td>Plasma, saliva, GCF</td>
</tr>
<tr>
<td>Carotenoids (vitamin A)</td>
<td>Chain breaking (scavenging)</td>
<td>Water soluble</td>
<td>Plasma, saliva, GCF</td>
</tr>
<tr>
<td>Albumin</td>
<td>Preventative (binds metal ions)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Chain breaking (scavenging)</td>
<td>Lipid soluble</td>
<td>Plasma</td>
</tr>
<tr>
<td>Caeruloplasmin</td>
<td>Preventative (binds metal ions)</td>
<td>Water soluble</td>
<td>Plasma, GCF</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Preventative (binds Fe²⁺ ions)</td>
<td>Water soluble</td>
<td>Plasma, saliva, GCF</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Chain breaking (scavenging)</td>
<td>Water soluble</td>
<td>Plasma, saliva, GCF</td>
</tr>
<tr>
<td>Reduced glutathione (including cysteine)</td>
<td>Substrate for enzyme GSH-Px</td>
<td>Water soluble</td>
<td>Plasma, alveolar lining fluid of lungs, GCF</td>
</tr>
</tbody>
</table>

CSF = cerebrospinal fluid.
TOCOPHEROLS (VITAMIN E)

Alpha-tocopherol is located within cell membrane phospholipids and is a major chain breaking antioxidant but has limited mobility, which restricts its efficacy. However, many reactive oxygen species are generated in aqueous solution, particularly those from phagocytes and the vascular endothelium, and the role of α-tocopherol in the pathogenesis of the periodontal diseases is thus likely to be a minor one. In one study no differences were detected in plasma vitamin E concentrations in patients with and without periodontal disease, and in another, its prostaglandin inhibitory properties were credited for reducing periodontal inflammation.

URIC ACID

Uric acid is a relatively powerful scavenging antioxidant of water soluble radicals, such as hypochlorous acid and singlet oxygen. It can also bind copper and iron ions and increased concentrations of uric acid breakdown products are reported in patients with rheumatoid arthritis. Reaction of uric acid with some radicals (for example, OH) can produce uric acid radicals, but these are easily removed by ascorbate. It has recently been reported that uric acid is the major (>70%) antioxidant in saliva and while it is also found in GCF, its antioxidant contribution to GCF has not been investigated.

CYSTEINE AND REDUCED GLUTATHIONE

Glutathione is an essential tripeptide with many important functions. In its reduced form (GSH) glutathione is an important antioxidant (radical scavenger), a property bestowed upon it by its central thiol containing cysteine amino acid. It is also regarded as a pivotal molecule to the immune system, especially for regulation of IL-2 dependent T-lymphocyte proliferation. The role of reduced glutathione (GSH) in the regulation of pro-inflammatory cytokines is of great potential importance in periodontal disease. There is evidence that increasing cytosolic cysteine (and thus GSH) concentrations of monocytes and macrophages (using a synthetic form of cysteine called N-acetylcysteine) blocks hydrogen peroxide mediated activation of NF-κB (fig 5), and thus production of pro-inflammatory cytokines by this route. Cytokines, such as TNFα, IL-1β and IL-6, are associated with the activation of bone resorbing processes and IL-8 is reported to stimulate polymorphonuclear leucocyte activity. Normally, intracellular concentrations of GSH are high (0.1–10 mM), but extracellular fluid concentrations are low (2 μM in human plasma). However, local production of GSH has been reported in alveolar lining fluid at very high concentrations of 400 μM, with concentrations being raised in smokers and deficient in patients with pulmonary fibrosis and acute respiratory distress syndrome.

Certain putative periodontopathogens are capable of metabolising L-cysteine or degrading GSH to form hydrogen sulphide within the periodontal pocket. The formation of hydrogen sulphide within the periodontal pocket is toxic to mammalian cells by inactivating cytochrome oxidase and is also reported to inhibit catalase. It can be seen how the degradation of cysteine by oral microflora may also prevent the inhibition of NF-κB mediated production of pro-inflammatory cytokines in the periodontal environment, thereby increasing the risk of cytokine related tissue damage.

THE CONCEPT OF TOTAL ANTIOXIDANT CAPACITY

As ROS and antioxidant defence mechanisms seem to act in concert rather than alone, an example being the re-cycling of α-tocopherol by vitamin C, studies of individual antioxidants in relation to inflammatory disease may not necessarily yield useful information. Research is now being directed towards assays for total antioxidant capacities of biological fluids, while not forgetting the importance of the constituent antioxidant compounds. Wayner et al have reported the measurement of total peroxyl radical trapping parameter (TRAP assay), which is sensitive to all known chain breaking antioxidants but is a complex and time consuming assay to perform. Our group has recently reported a rapid and simple enhanced chemiluminescence (ECL) assay for total antioxidant quantification in biological fluids including serum, saliva and GCF. Initial data have revealed a reduced salivary total antioxidant concentration in patients with periodontitis relative to those with periodontal health, with no differences in serum concentrations. Guarnieri et al investigated GCF superoxide scavenging capacity in 14 patients with adult periodontitis and 16 healthy controls. They found no difference between test and control groups. The differences in results may be explained by the fact that in this study GCF was collected by a crevice washing technique that involved 12 washings with a microsyringe and samples were centrifuged and stored at −20°C prior to assay. It is likely that the repeated washing and centrifugation would have resulted in oxidation of several antioxidant components by the time of assay. Furthermore, data are available demonstrating that storage of fluid samples at −20°C results in a loss of antioxidant activity with time, and that storage should be by immersion in liquid nitrogen to prevent oxidation.

A local antioxidant response found in GCF and also in saliva but not in serum, has been identified as a low molecular weight (<10 kilodaltons) thiol, and this response has been reproduced from the cytosol of anaerobically stimulated neutrophils and mimicked using L-cysteine and cysteamine. It seems logical that polyunsaturated fatty acids were thought of as powerful intracellular antioxidants in view of their high potential for radical generation. However, the discovery of high GSH concentrations within alveolar lining fluid and the detection of a low molecular weight thiol in GCF raises the possibility that the production of antioxidant thiols at vulnerable epithelial surfaces is an important defence mechanism.
against unwanted ROS mediated damage (whether polymorphonuclear leucocyte mediated or from exogenous sources) that is common to several organs. Considerable work is needed in this area to elucidate the true identity of the GCF antioxidant reported and to investigate the presence of similar thiol antioxidants at high concentrations in anatomical sites that are exposed to similar sources of potential damage, such as the cervical epithelium.

Conclusions

There are many similarities between the host-parasite interactions that characterise periodontitis and diseases affecting other areas of the body, such as inflammatory lung disease and rheumatoid arthritis. In the lungs and periodontal tissues, the external environment is separated from the internal connective tissues by a delicate epithelial barrier, behind which large scale polymorphonuclear leucocyte responses are seen in reaction to various bacterial activities. While the role of the polymorphonuclear leucocyte is primarily a protective one, host tissue damage can result indirectly from over exuberant polymorphonuclear leucocyte and monocyte responses as well as directly from the colonising pathogens themselves. Reactive oxygen species produced by inflammatory and immune cells have been associated with significant tissue damage. To combat such damage the body possesses a variety of antioxidant defence mechanisms, whose role is to protect vital cell and tissue components from radicals of host cell as well as parasitic origin. Study of local antioxidant systems is likely to yield valuable information about the pathogenesis of certain diseases, and such antioxidants should be studied when acting in concert as well as in individual systems. The balance between antioxidant defence and repair systems, and pro-oxidant mechanisms of cell damage may be tipped in favour of tissue destruction either by increases in radical production or by a lowered antioxidant defence. While the former situation has been demonstrated in periodontitis, the latter has not, as the technology has not been available to measure total antioxidant defence in GCF until recently. Production of high concentrations of the antioxidant thiol, reduced glutathione (GSH), from the internal connective tissues of the lung where delicate epithelial tissues are exposed to free radical attack. GSH is dependent upon the amino acid cysteine for its antioxidant activity, for IL-2 dependent T-cell functions and also for other vital anti-inflammatory mechanisms that rely upon preventing NF-xB translocation to defence cell nuclei. A local low molecular weight thiol has also been detected in GCF, which seems to contribute substantially to its overall antioxidant capacity. The kinetics of light recovery for this antioxidant, in the enhanced chemiluminescence assay used for its detection, can be reproduced by L-cysteine which may be of epithelial cell as well as polymorphonuclear leucocyte origin. Considerable work is needed to evaluate further local epithelial defence systems, such that the important anti-inflammatory mechanisms, in addition to anti-oxidant activities of thiols such as reduced glutathione and cysteine, can be fully understood. If the GCF thiol is cysteine or GSH, these data together with those from alveolar lining fluid 31 may lead to the development of new therapeutic pathways for inflammatory disease using cysteine and GSH preserving drugs such as N-acetylcysteine, or indeed, other NF-xB inhibitors.

I would like to express my sincere thanks to Dr J B Matthews for reading this manuscript and for his very helpful comments.


32 Morrow JD, Hill KE, Burke RF, Namour TM, Bads KD, Roberts SB. A series of prostaglandin F2alpha-like compounds are produced in vivo in humans by a non-cytochrome P450, free-radical-catalysed mechanism. Proc Natl Acad Sci USA 1990;87:933–7.


